PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
K 2585		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 98/ 05110	12/08/1998	20/08/1997

Applicant

DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG ..et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. -

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

The present invention relates to a protease-related protein, which plays a role in the keratinization of hair a DNA encoding the same and a process for the preparation thereof. Furthermore, this invention concerns the use of the DNA and the protein as well as antibodies directed against the protein and antagonistic substances.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/05110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K16/18 A61K48/00 C07K14/47 G01N33/68
A61K39/395 C12N1/21 A61K38/17 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUROOKA ET AL: "Rescue of the hairless phenotype in nude mice by transgenic insertion of the wild-type Hfh11 genomic locus" INTERNATIONAL IMMUNOLOGY, vol. 8, no. 6, 1996, pages 961-966, XP002093572 cited in the application see the whole document ---	1-15
A	NOZAKI ET AL: "The complete sequence of the gene encoding mouse cytokeratin 15" GENE, vol. 138, 1994, pages 197-200, XP002093573 cited in the application see the whole document ---	10,11,15
	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1999	09/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Jansen, K-S

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WINTER ET AL: "Sequence and Expression of Murine Type I Hair Keratins mHa2 and mHa3" EXPERIMENTAL CELL RESEARCH, vol. 212, 1994, pages 190-200, XP002093574 cited in the application see the whole document	10,11,15

PATENT COOPERATION TREATY

EINGEGANGEN
17. NOV. 1999
PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SCHÜSSLER, Andrea
Huber & Schüssler
Truderinger Strasse 246
D-81825 München
ALLEMAGNE

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	15. 11. 99
Applicant's or agent's file reference K 2585	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/05110	12/08/1998	20/08/1997

Applicant
DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG ..et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

GP

PATENT COOPERATION TREATY

REC'D 18 NOV 1999
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
K 2585		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/05110	12/08/1998	20/08/1997
International Patent Classification (IPC) or national classification and IPC		
C12N15/12		
Applicant		
DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG ..et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/03/1999	15. 11. 99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Julia, P Telephone No. +49 89 2399 8410

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/05110

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

   Description, pages:

   1-20 as originally filed

   Claims, No.:

   1-15 as originally filed

   Drawings, sheets:

   1/2-2/2 as originally filed

2. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

   see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has

been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 7-15
	No:	Claims 1-6
Inventive step (IS)	Yes:	Claims 7-15
	No:	Claims 1-6
Industrial applicability (IA)	Yes:	Claims 1-15
	No:	Claims

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. Additional remarks to item I :

The Applicant has filed with its letter dated 18.12.98 (received on 19.12.98) a Sequence Listing comprising pages 1-4 and SEQ ID No.: 1 and 2.

2. Additional remarks to item II :

The priority documents pertaining to the present application were not available at the time of establishing this international preliminary examination report (IPER). Hence, the current assessment is based on the assumption that all claims enjoy priority rights from the filing date of the priority document (20.08.97).

3. Additional remarks to item V :

The present application discloses a gene which is regulated by the gene product of the known "whn" gene (a transcription factor which also regulates the expression of the known keratins Ha3 and CK15. If this transcription factor is lacking the genes of Ha3 and CK15 are underexpressed, whereas the gene disclosed in the application is overexpressed. In fact, basis for isolating these genes by the "representational difference analysis" (RDA) method), wherein said gene codes for a protease-related protein (PVP) (related to a protease of the kallikrein family) (figure 1 and SEQ ID No.: 1, DNA and amino acid sequences and SEQ ID No.: 2 amino acid sequence). This protein is said to be involved in the keratinization of hair and the application explicitly claims uses of such protein (with and/or without Ha3 and/or CK15, antibodies, antisense, DNA etc...) for regulating the keratinization of hair, wherein a positive regulation implies the inhibition of PVP (by antibody, antisense, etc...) and a negative regulation implies the presence or administration of PVP. However, there is no example demonstrating any particular relation to or effect on keratinization of hair.

None of the documents cited in the International Search Report (IPER) discloses the specific amino acid sequence or nucleotide sequence encoding the protease-related protein of the present application (SEQ ID No.: 1-2). However, in view of the actual wording of the claims and in particular the reference to "..differing .. by one or more amino acids.." (see paragraph (i) below under "Additional remarks to item VIII"), the IPEA considers that the subject matter of claims 1-2 is anticipated by known proteases of the kallikrein family (in particular from the chimotrypsin protease family) and that for these products the embodiments of claims 3-6 are also well-known. Thus, the subject matter of claims 1-6 does not fulfil the requirements of Articles 33 (2) and 3) PCT.

The documents cited in the International Search Report (ISR) are concerned with the whn gene and its effects (H. Kurooka et al., Int. Immunol. 1996, Vol. 8 (6), pages 961-966, **D1**) as well as the specific CK15 gene/protein (M. Nozaki et al., Gene 1994, Vol. 138, pages 197-200, **D2**) and the Ha3 and Ha2 genes/proteins (H. Winter et al., Exp. Cell Res. 1994, Vol. 212, pages 190-200, **D3**). None of said documents suggests let alone discloses and characterizes the **specific** protease-related of the present application and/or the uses referred in claims 7-15, which are thus considered to be novel and inventive (Articles 33 (2) and (3) PCT).

4. Additional remarks to item VII :

i) page 18 first line of example 13 refers to "a fusion protein of example 1", whereas fusion proteins are only disclosed in example 2. The same error is found on page 19 line 12.

ii) claim 12 wrongly refers to a "certification of hair" instead of a "keratinization of hair".

5. Additional remarks to item VIII :

The following objections are also raised under **Article 6 PCT** concerning the clarity of the claims :

i) the IPEA considers that by using the wording "... or an amino acid sequence differing therefrom by one or more amino acids ..." the actual scope of claim 1 is completely open and ambiguous. In fact, any "protease-related protein" known from the prior art can be easily obtained from the amino acid sequence of figure 1 of the present application by a suitable number (and type) of changes. The same objection applies to the subject matter of claim 2(a) by reference to "... a DNA differing therefrom by one or more base pairs ...". In addition, the IPEA considers that general references to "hybridization" without clearly specifying the conditions used and without any further requirement of the nucleic acid to be hybridized are also ambiguous and not clear.

ii) the subject matter of claim 6 is directed to general antibodies directed against the claimed protease-related protein of claim 1. The IPEA considers that almost any protein contains portions or domains (as well as small, arbitrary peptides that can be also used as haptens), which can have a significant homology to similar domains of other completely unrelated proteins (apart from the presence of said domains). These domains do comprise

immunoepitopes which can elicit antibodies that (more or less) cross-react with these unrelated proteins. Thus, antibodies directed to other protease proteins but which (cross)react with the protease-related protein of the present application would be novelty destroying for the subject matter of said claim 6.

In addition, the attention of the Applicant is drawn to the fact that the production of "specific or selective" antibodies requires the identification of "specific, selective or unique" immunoepitopes, the production of antibodies directed against said immunoepitopes, the demonstration that said antibodies are able to successfully react against the whole protein (containing such immunoepitopes. It could well be that structural constrains hinder the identification of said immunoepitopes when present in the whole protein) and that they are actually "specific" (and relevant) of said protein. Therefore, the IPEA considers that as far as said "specific" epitopes are not explicitly disclosed in the description or else the production of said "specific" antibodies is not clearly exemplified in the description, such a subject matter would be only worded in terms of the result to be achieved and it can not be seen as being **fully** (i.e. formally and technically) supported by the description as required by Article 6 PCT. Furthermore, in view of the significant homology between the claimed protease-related protein and other known proteases and in particular proteases of the kallikrein family, the IPEA considers that the "specific" epitopes should be clearly and explicitly defined in those claims. Thus, the subject matter directed to a general "antibody" or a general "antibody specific" for the claimed protease-related protein, namely claim 6 does not fulfil all these requirements.

iii) by using the wording "and" and "as well as" the wording of claim 7 implies the simultaneous presence of three different products for the claimed use, namely the protease-related protein, the corresponding DNA and the antibody.

iv) the attention of the Applicant is also drawn to the fact that the subject matter of different "use-type claims" embraces possible methods for treatment of the human or animal body and thus, they would be excluded from examination by Article 34(4)(a)(i) PCT in combination with Rule 67(iv) PCT. Furthermore, for such a subject matter no unified criteria exist in PCT for the assessment whether it is industrially applicable or not. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject matter of claims to the use of a compound in medical treatment, but will allow, however, claims to a known

compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

v) according to Article 6 PCT in combination with Rule 6.3 PCT the claims shall define the matter for which protection is sought in terms of technical features. The IPEA considers that a peptide, polypeptide, protein, oligonucleotide, gene, etc.. being chemical products must be clearly and unambiguously characterized by their amino acid and/or nucleic acid sequences, i.e. by reference to their specific SEQ ID No. The characterization of a product only by the desired function or by an arbitrary abbreviation, such as Ha3 and CK15 (unless said abbreviations are well-known to the skilled person and do not allow any ambiguity in their interpretation) and without any real technical meaning does not fulfill the requirements of said Article 6 PCT in combination with Rule 6.3 PCT. Thus, the subject matter of claims 10-11 and 15 does not meet these requirements.

In this respect, essential technical elements of claim 10 are the "substances which inhibit the proteins Ha3 and/or CK15". It is not clear to the IPEA whether such substances are well-known and easily available to the skilled person, in particular for all types of inhibitors, namely the ones (i) inhibiting Ha3 but not CK15, (ii) inhibiting CK15 but not Ha3 or (iii) inhibiting both Ha3 and CK15. None of these substances has been referred let alone characterized in the present description.

vi) the subject matter of claim 13 is ambiguous. According to the claim, the disclosed protease-related protein must be present "in the form of a substance inhibiting it". It is not clear whether said inhibition refers to the possible "protease activity" or to another non-disclosed activity. Furthermore, it is not clear whether it intends to require the simple presence of an inhibitor (such as seems to be implied by claim 14 and page 5 of the description), the presence of an inhibitor (linked, associated, etc...) together with the protease-related protein, or else the presence of a modified or altered form of the protease-related protein with the ability to inhibit the (whatever !!) activity of the "wild-type" protease-related protein.

vii) furthermore, the IPEA considers that the present application does not provide any example or demonstration that the disclosed protease-related protein is actually involved in the (positive and/or negative) regulation of the keratinization of hair. Thus, the IPEA considers that there is no (technical) support for the specific uses of claims 7 to 15.

The demand must be filed directly with the competent International Preliminary Examining Authority; or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ _____

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: K 2585 Wd

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/EP98/05110	12. 08.1998	20.08.1997

Title of invention: Protease-Related Protein

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Deutsches Krebsforschungszentrum
Stiftung des öffentlichen Rechts
Im Neuenheimer Feld 280
69120 Heidelberg
DE

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(i.e. country)* of nationality: DE

State *(i.e. country)* of residence: DE

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

DEAR, Terence N.
Wundstr. 31
69123 Heidelberg
DE

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: DE

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BOEHM, Thomas
Lucas-Cranach-Str. 4
69126 Heidelberg
DE

State *(i.e. country)* of nationality: DE

State *(i.e. country)* of residence: DE

☐ Further applicants are indicated on a continuation sheet.

ernational application No.
PCT/EP98/05110

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Dr. Andrea Schüßler

HUBER & SCHÜSSLER
Patentanwälte · Patent Attorneys
Truderinger Straße 246 · 81825 München
Tel. 089/42 72 47 48 · Fax 089/42 72 47 49

Telephone No.:

Facsimile No.:

Teleprinter No.:

[ ] Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

(i) [ ] to start the international preliminary examination on the basis of the international application as originally filed.

(ii) [ ] to take into account the amendments under Article 34 of

- [ ] the description (amendments attached).
- [ ] the claims (amendments attached).
- [ ] the drawings (amendments attached).

(iii) [ ] to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) [ ] to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) [ ] to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

[X] The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)* except .....
.....
.....
.....

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above.)

ernational application No.
PCT/EP98/05110

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

			For International Preliminary Examining Authority use only: received	not received
1. amendments under Article 34				
description	:	sheets	☐	☐
claims	:	sheets	☐	☐
drawings	:	sheets	☐	☐
2. letter accompanying amendments under Article 34	:	sheets	☐	☐
3. copy of amendments under Article 19	:	sheets	☐	☐
4. copy of statement under Article 19	:	sheets	☐	☐
5. other *(specify)*:	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ fee calculation sheet
5. ☒ other *(specify)*: cheque

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Dr. Andrea Schüßler

Munich, March 17, 1999

Patent Attorney

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
K 2585		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 98/ 05110	12/08/1998	20/08/1997

Applicant

DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG ..et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. -

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

The present invention relates to a protease-related protein, which plays a role in the keratinization of hair a DNA encoding the same and a process for the preparation thereof. Furthermore, this invention concerns the use of the DNA and the protein as well as antibodies directed against the protein and antagonistic substances.

INTERNATIONAL SEARCH REPORT

International Application No
T/EP 98/05110

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUROOKA ET AL: "Rescue of the hairless phenotype in nude mice by transgenic insertion of the wild-type Hfh11 genomic locus" INTERNATIONAL IMMUNOLOGY, vol. 8, no. 6, 1996, pages 961-966, XP002093572 cited in the application see the whole document ---	1-15
A	NOZAKI ET AL: "The complete sequence of the gene encoding mouse cytokeratin 15" GENE, vol. 138, 1994, pages 197-200, XP002093573 cited in the application see the whole document ---	10,11,15
	-/--	

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1999	09/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Jansen, K-S

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International Application No
T/EP 98/05110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WINTER ET AL: "Sequence and Expression of Murine Type I Hair Keratins mHa2 and mHa3" EXPERIMENTAL CELL RESEARCH, vol. 212, 1994, pages 190-200, XP002093574 cited in the application see the whole document	10,11,15

PATENT COOPERATION TREATY

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Applicant DEAR, Terence, N. et al	

1. The designated Office is hereby notified of its election made:

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(54) Title: PROTEASE-RELATED PROTEIN

(57) Abstract

The present invention relates to a protease-related protein which plays a role in the keratinization of hair, a DNA encoding the same and a process for the preparation thereof. Furthermore, this invention concerns the use of the DNA and the protein as well as antibodies directed against the protein and antagonistic substances.

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(54) Title: PROTEASE–RELATED PROTEIN

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The present invention relates to a protease–related protein which plays a role in the keratinization of hair, a DNA encoding the same and a process for the preparation thereof. Furthermore, this invention concerns the use of the DNA and the protein as well as antibodies directed against the protein and antagonistic substances.

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Protease-Related Protein

The present invention relates to a protease-related protein, a DNA encoding the same and a process for the preparation thereof. In addition, this invention concerns the use of the DNA and the protein as well as antibodies directed against the protein.

A hair anomaly is frequently due to an impaired keratinization of hair. It is known from investigations made with naked mice that the gene product of a gene referred to as whn is important for the keratinization of hair. This gene product is a transcription factor. However, its target genes are not known. In so far, it is not possible to interfere with the keratinization of hair. However, this would be desirable, particularly if the keratinization of the hair is impaired.

Therefore, it is the object of the present invention to provide a product by which the keratinization of hair can be investigated and optionally be regulated.

According to the invention this is achieved by the subject matters defined in the claims.

Thus, the subject matter of the present invention is represented by a protease-related protein, the protein comprising the amino acid sequence of fig. 1 or an amino acid sequence differing therefrom by one or more amino acids.

The present invention is based on the applicant's finding that the gene product of the whn gene is responsible for regulating the expression of at least three genes. Two of these genes code for the known keratins Ha3 (cf. Winter, H. et al., Exp. Cell Res. 212 (1994), 190-200) and CK15 (cf. Nozaki, M. et al., Gene 138 (1994), 197-200), respectively.

The third gene codes for a protein which has homologies with respect to a protease of the kallikrein family, optionally a protease activity, but differs from a known protease of the kallikrein family on the DNA level by hybridization under normal conditions. Such a protein has the amino acid sequence of fig. 1 or an amino acid sequence differing therefrom by one or more amino acids. Furthermore, the applicant has found that when the gene product of the whn gene is lacking the genes of Ha3 and CK15 are underexpressed whereas the gene of the above protein is overexpressed.

The above protein is referred to as "protease-related protein" (PVP) in the present invention.

Another subject matter of the present invention relates to a nucleic acid coding for a (PVP). This may be an RNA or a DNA. The latter may be a genomic DNA or a cDNA, for example. Preferred is a DNA comprising the following:

(a) the DNA of fig. 1 or a DNA differing therefrom by one or more base pairs,
(b) a DNA hybridizing with the DNA of (a), or
(c) a DNA related to the DNA of (a) or (b) via the degenerated genetic code.

The expression "hybridizing DNA" refers to a DNA which hybridizes with a DNA of (a) under normal conditions, particularly at 20°C below the melting point of the DNA.

A section of the DNA of fig. 1 was deposited with the DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* [German-type collection of microorganisms and cell cultures]) as pRDA2-1a under DSM 11522 on April 23, 1997.

A DNA according to the invention is described below in the form of a cDNA. It stands as an example for every DNA falling under the present invention.

For the production of a cDNA according to the invention it is favorable to isolate mRNA from skin cells of whn(+/+) mice and nu/nu(whn(-/-)) mice, respectively, to transcribe the mRNA into cDNA and subject the cDNA to a "representational difference analysis" (RDA) method (cf. Hubank, M. and Schatz, D., Nucleic Acids Research 22 (1994), 5640-5648) so as to identify that cDNA which is underexpressed and overexpressed, respectively, in nu/nu mice as compared to whn(+/+) mice. In particular the latter cDNA represents a cDNA according to the invention.

A cDNA according to the invention may be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli, these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. For the expression in yeast, pY100 and Ycpad1 have to be mentioned as examples, while for the expression in animal cells, e.g. pKCR, pEFBOS, cDM8 and pCEV4 have to be indicated. For the expression in insect cells, the baculo virus expression vector pAcSGHisNT-A is especially suitable.

The person skilled in the art is familiar with suitable cells to express a cDNA according to the invention, which is present in an expression vector. Examples of such cells comprise the E. coli strains HB101, DH1, x1776, JM101, JM 109, BL21 and SG 13009, the yeast strain *saccharomyces cerevisiae* and the animal cells Ltk, 3T3, FM3A, CHO, COS, Vero and HeLa as well as the insect cells sf9.

The person skilled in the art knows in which way a cDNA according to the invention has to be inserted in an expression vector. He is also familiar with the fact that this DNA can be inserted in connection with a DNA coding for another protein and peptide, respectively, so that the

cDNA according to the invention can be expressed in the form of a fusion protein.

In addition, the person skilled in the art is familiar with conditions of cultivating transformed cells and transfected cells, respectively. He also knows processes serving for isolating and purifying the protein expressed by the cDNA according to the invention. Thus, such a protein which may also be a fusion protein represents a subject matter of the present invention as well.

Another subject matter of the present invention relates to an antibody directed against an above protein and fusion protein, respectively. Such an antibody may be prepared by conventional processes. It may be polyclonal and monoclonal, respectively. For its preparation it is favorable to immunize animals, particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody, with an above (fusion) protein or fragments thereof. Further boosters of the animals can be effected with the same (fusion) protein or fragments thereof. The polyclonal antibody may then be obtained from the animals' serum and yolk, respectively. For the monoclonal antibody, spleen cells from the animals are fused with myeloma cells.

The present invention enables to investigate the keratinization of hair. (PVP) can be detected in cells, particularly skin cells, by means of an antibody according to the invention. A relation between (PVP) and the keratinization of hair can be established. Moreover, it is possible to detect by means of a (PVP) according to the invention an autoantibody directed against this protein. Both detections may be made by conventional processes, particularly a Western blot, an ELISA, an immunoprecipitation or by immunofluorescence. Furthermore, the expression of the gene coding for (PVP) can be detected by a nucleic acid according to the invention, particularly

a DNA and primers derived therefrom. This detection can be carried out as usual, particularly in a Southern blot.

In addition, the present invention is suited to interfere in regulating fashion with the keratinization of hair. This regulation may be positive or negative. A positive regulation is understood to mean one by which an impaired keratinization of hair can be encountered. A negative regulation would exist if a normal or strong keratinization of hair was reduced.

For a positive regulation of the keratinization of hair it is an obvious thing to use (PVP) in the form of a substance inhibiting it. This substance may be an antibody according to the invention. Furthermore, it may be an anti-sense oligonucleotide which is suited for the expression inhibition of the gene coding for (PVP). Moreover, the substance may be a substance which has an antagonistic effect with respect to (PVP). It may be advantageous to use several substances. It may be especially favorable to use additionally one or more of the proteins Ha3 and CK15 as such or in the form of nucleic acids expressing them.

For a negative regulation of the keratinization of hair, it is an obvious thing to use (PVP) as such or in the form of a nucleic acid expressing it. It may be advantageous to use additionally one or more of the proteins Ha3 and CK15 in the form of substances inhibiting them. Such substances may be antibodies directed against Ha3 and CK15, respectively, or anti-sense oligonucleotides, which are suited for the expression inhibition of the genes coding for Ha3 and CK15, respectively. The substances may also be those which have an antagonistic effect with respect to Ha3 and CK15, respectively.

Another subject matter of the invention relates to a product which is suited for regulating the keratinization of hair. For the composition of such a product the above

statements made on the positive and negative regulations of the keratinization of hair apply correspondingly.

Thus, the present invention represents a major contribution to the understanding of the keratinization of hair and a possible regulating interference.

Brief description of the drawing:

Fig. 1 shows the base sequence of a cDNA according to the invention as well as the amino acid sequence, derived therefrom, of a (PVP) according to the invention.

The present invention is explained by the below examples.

Example 1: Preparation of a cDNA according to the invention

A cDNA according to the invention was prepared according to the "representational difference analysis" (RDA) method. This method comprises the isolation of mRNA from skin cells of whn(+/+) mice and nu/nu mice, respectively, the transcription of the mRNA into cDNA, and the differentiation of the cDNA so as to identify that which is underexpressed and overexpressed, respectively, in nu/nu mice. In particular, the latter represents a cDNA according to the invention.

A) Sequence of the oligonucleotide adaptors

The following oligonucleotide adaptor pairs were required for the RDA:

R-Bgl-12: 5'-GATCTGCGGTGA-3'
R-Bgl-24: 5'-AGCACTCTCCAGCCTCTCACCGCA-3'

R-Bgl-12: 5'-GATCTGTTCATG-3'
R-Bgl-24: 5'-ACCGACGTCGACTATCCATGAACA-3'

N-Bgl-12: 5'-GATCTTCCCTCG-3'
N-Bgl-24: 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

B) Preparation of poly A-mRNA from the tissues to be compared with one another

RNA was initially obtained from the skin of whn(+/+) mice and nu/nu mice, respectively, according to the "single step RNA extraction" method (Chomczynski and Sacchi, 1987). Thereafter, the poly A-mRNA fractions from the two RNA populations were isolated by means of dynabeads oligo(dT) according to the corresponding protocol from the Dynal company.

C) Synthesis of double-stranded cDNA

The "ribo clone cDNA synthesis kit" from the company of Promega was used for the synthesis of double-stranded whn(+/+) cDNA and nu/nu cDNA, respectively. 4 µg of poly A-mRNA were used each to obtain about 2 µg cDNA.

D) Difference analysis

1. Restriction digest of the double-stranded cDNAs

   a) About 2 µg of each cDNA were digested in a 100 µl reaction batch by the restriction endonuclease Dpnl1 at 37°C for 2 h.

   b) The reaction solutions were then extracted twice with a phenol/chloroform mixture (1:1) and once with 100 % chloroform.

   c) The DNA included in the aqueous phases of the two reaction batches was admixed with 2 µg glycogen,

50 µl 10 M ammonium acetate and 650 µl 100 % ethanol each and precipitated on ice for 20 min.

After 14 minutes of centrifugation at 4°C and with 14000 rpm, the supernatant was discarded and the DNA pellet was washed with 70 % ethanol. After another centrifugation and removal of the alcoholic phase, the dried DNA was resuspended in 20 µl TE buffer.

2. Ligation of the cDNAs to the R-Bgl oligonucleotide adaptor pair

a) A reaction vessel was used to combine the following:
20 µl cut cDNA (total reaction batch from item D)1c)
8 µg R-Bgl-24
4 µg R-Bgl-12
6 µl 10 x ligase buffer
x µl water
57 µl final volume

b) The reaction batch was heated in a thermocycler (Peltier Thermocycler PTC-200, MJ Research) to 50°C, kept at this temperature for 1 min and then cooled again to 10°C in the course of one hour (ramp rate: 0.1°C/9 sec).

c) After adding 3 µl T4 DNA ligase (1 U/µl), the mixture was incubated at 16°C overnight.

3. Synthesis of "representations" of the cDNA populations to be compared with one another

a) For generating what is called "representations" of the ligated cDNAs, the volume of the ligation batches from item 2c) was initially supplemented

by the addition of 140 µl water each to give 200 µl.

Then, 30 reactions of 200 µl each were prepared from this dilute solution per cDNA population (whn(+/+) skin and nu/nu skin, respectively).

The following reactants were added successively to such a batch:
143 µl water
20 µl 10x PCR buffer
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl R-Bgl-24 (1 µg/µl)
4 µl dilute ligation batch

b) PCR:
3 min: 72°C
addition of 1 µl Taq-DNA polymerase (5 U/µl)
20 x: 5 min: 95°C
3 min: 72°C
finally: cooling to 4°C.

c) For preparing the reaction solutions, 4 reaction batches each were combined in a vessel.
extraction: 2 x with 700 µl phenol/chloroform (1:1) each, 1 x with chloroform 100 %;
precipitation: addition of 75 µl 3 M Na-acetate solution (pH 5.3) and 800 µl 2-propanol to each reaction vessel, 20 min on ice.
centrifugation: 14 min, 14000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70 % and resuspension in such an amount of water that a concentration of 0.5 µg/µl resulted.

4. Restriction digest of the "representations"

a) For removing the R-Bgl oligonucleotide adaptors, 300 µg of each representation (whn(+/+) skin and nu/nu skin, respectively) were subjected to a restriction digest. Following the addition of the below reactants, incubation was carried out at 37°C for 4 h:
600 µl cDNA representation (0.5 µg/µl)
140 µl 10 x DpnII buffer
100 µl DpnII (10 U/µl)
560 µl water.

b) The restriction digest batch was distributed to 2 vessels prior to its preparation.
Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %;
precipitation: addition of 70 µl 3 M Na-acetate (pH 5.3), 700 µl 2-propanol to each vessel, 20 min on ice;
centrifugation: 14 min, 14000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70 % and resuspension in such an amount of water that a concentration of 0.5 µg/µl resulted.

The resulting DpnII-digested whn(+/+) skin-cDNA representation represented the driver-DNA population to be used in the subtractive hybridization.

5. Synthesis of the tester DNA population

a) 20 µg of the nu/nu skin-cDNA representation digested with DpnII (= tester DNA) were separated electrophoretically in a TAE gel:
40 µl tester DNA (0.5 µg/µl)
50 µl TE buffer
10 µl 10 x loading buffer

were applied to a 1.2 % agarose TAE gel. A voltage was applied to the gel until the bromophenol blue component of the loading buffer had migrated about 2 cm.

b) Thereafter, the bands containing the representation DNA were cut out of the gel and eluted by means of the "agarose gel DNA extraction kit" from the company of Boehringer Mannheim.

The DNA extracts were purified, so that a total of 60 µl of solution was obtained. The concentration of this solution was evaluated by electrophoresis of 5 µl in a 1 % agarose gel.

c) Finally, the tester DNA was ligated with the J oligonucleotide pair:
2 µg tester DNA eluate
6 µl 10 x ligase buffer
4 µl J-Bgl-24 (2 µg/µl)
4 µl J-Bgl-12 (1 µg/µl)
x µl water
57 µl final volume

d) Transfer of the reaction batch into thermocycler:
1 min: 50°C
cooling to 10°C within 1 h (ramp rate: 0.1°C/9 sec).

e) After adding 3 µl T4 DNA ligase (1 U/µl), incubation at 16°C overnight.

f) Adjusting the concentration of the tester DNA to approximately 10 ng/µl by the addition of 120 µl water.

6. Subtractive hybridization

a) 80 µl of driver DNA (40 µg) from step 4. and 40 µl (0.4 µg) of dilute tester DNA from step 5., ligated with J oligonucleotides, were combined in a reaction vessel and extracted 2 x with phenol/chloroform (1:1) and once with chloroform 100 %.

b) Precipitation by addition of 30 µl of 10 M ammonium acetate, 380 µl of ethanol 100 %; -70°C for 10 min.
Centrifugation: 14 min, 14000 rpm, 4°C.
Then: 2 x washing of the pellet with ethanol 70 %, short centrifugation after every wash step; drying of the DNA pellet.

c) The DNA was resuspended in 4 µl EE x3 buffer (30 mM EPPS, pH 8.0, at 20°C (Sigma company), 3 mM EDTA) - accompanied by pipetting off and on for about 2 min, then heating to 37°C for 5 min, short "vortexing" and finally combining the solution again on the vessel bottom by centrifugation. The solution was eventually covered with a layer consisting of 35 µl of mineral oil.

d) Transfer of the reaction batch into thermocycler:
5 min: 98°C,
cooling to 67°C and immediate addition of 1 µl 5 M NaCl to the DNA,
20 h of incubation at 67°C.

7. Synthesis of the first difference product

a) After removing the mineral oil as completely as possible, the DNA was diluted step-wise:
   1. addition of 8 µl TE (+ 5 µg/µl yeast RNA)
   2. addition of 25 µl TE - thereafter thorough mixing
   3. addition of 362 µl TE - vortex.

b) 4 PCRs were prepared for every subtractive hybridization. Per reaction:
   127 µl water
   20 µl 10 x buffer
   20 µl 2 mM dNTPs
   5 µl 25 mM Mg chloride
   20 µl dilute hybridization solution (from step 7a))

c) PCR program:
   3 min: 72°C
   addition of 1 µl Taq DNA polymerase (5 U/µl)
   5 min: 72°C
   addition of 2 µl primer J-Bgl-24 (1 µg/µl)
   10 x: 1 min: 95°C
   3 min: 70°C
   finally: 10 min: 72°C, then cooling to room temperature

d) The 4 reaction batches were combined in a 1.5 ml vessel.
   extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100%.
   After the addition of 2 µg glycogen carrier:
   Precipitation with 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min on ice.
   Centrifugation: 14 min, 14000 rpm, 4°C.
   Washing of the DNA pellet with ethanol 70 %.

After drying of the DNA, resuspension in 40 µl water.

e) 20 µl of the resuspended DNA from d) were subjected to a "mung bean nuclease digest" (MBN):
20 µl DNA
4 µl 10 x mung bean nuclease buffer (NEB company)
14 µl water
2 µl mung bean nuclease (10 U/µl; NEB company)
35 min, 30°C.

The reaction was discontinued by the addition of 160 µl 50 mM Tris-HCl (pH 8.9) and 5 minutes of incubation at 98°C. Thereafter, the vessel was placed on ice up to the next step.

f) During the MBN incubation, 4 further PCRs were prepared (on ice):
127 µl water
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl J-Bgl-24 (1 µg/µl)
20 µl MBN-digested DNA.

g) PCR program:
1 min: 95°C
allowing to cool to 80°C, addition of 1 µl Taq DNA polymerase (5 U/µl),
18 x: 1 min: 95°C
3 min: 70°C,
finally: 10 min: 72°C; allowing to cool to 4°C.

h) The 4 PCR batches were combined in a vessel.
Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %.
Precipitation: 75 µl 3 M Na-acetate (pH 5.3), 800 µl 2-propanol, 20 min on ice.
Centrifugation: 14 min, 14000 rpm, 4°C.

Washing of the DNA pellet with ethanol 70 %.
Resuspension of the DNA in 100 µl water (resulting concentration: 0.5 µg/µl); the solution obtained in this way represented the first difference product.

8. Exchange of the oligonucleotide adaptors of the difference product

a) Removal of the oligonucleotide adaptors by restriction digest with DpnII:
40 µl difference product 1 (0.5 µg/µl)
30 µl 10 x DpnII buffer
15 µl DpnII (10 U/µl)
215 µl water

37°C for 2 h.

b) Preparing the reaction batch:
Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %.
Precipitation: 33 µl 3 M Na-acetate (pH 5.3), 800 µl ethanol 100 %, 20 min, -20°C.
Centrifugation: 14 min, 14000 rpm, 4°C.
Washing of the pellet in ethanol 70 % and resuspension in 40 µl water.

c) Ligation of the difference product to N-Bgl oligonucleotide adaptor pair
1 µl of the prepared DNA solution from step b) was diluted with 9 µl water to give a concentration of 50 ng/µl; 4 µl of this solution were used in the following reaction:
4 µl DpnII-digested difference product 1 (200 ng)
6 µl 10 x ligase buffer
2.5 µl N-Bgl-24 (3.5 µg/µl)
2 µl N-Bgl-12 (2 µg/µl)
42.5 µl water.

d) After the transfer of the reaction batch into thermocycler:
1 min: 50°C,
allowing to cool to 10°C within one hour (ramp rate: 0.1°C/9 sec).

e) After adding 3 µl T4 DNA ligase (1 µg/µl), incubation at 16°C overnight.

9. Synthesis of the 2nd difference product

The ligation batch from step 8e) was diluted by adding 100 µl water to a concentration of 1.25 ng/µl. 40 µl of this dilution (50 ng) were mixed with 80 µl driver DNA (see item 4.) and treated again according to steps 6. to 8. When the oligonucleotide adaptors (step 8.) were changed, the J-Bgl oligonucleotides were ligated in this case to the newly formed difference product 2.

10. Synthesis of the 3rd difference product

The concentration of the difference product 2 ligated with the J-Bgl oligos was reduced to a concentration of 1 ng/µl. 10 µl of this solution were diluted again with 990 µl water (+ 30 µg yeast-RNA), so that the concentration was then 10 pg/µl. The subtractive hybridization was carried out with 100 pg (10 µl) J-ligated difference product 2 and 40 µg (80 µl) driver DNA from step 4. As for the rest, the procedure was carried out as in the case of the 1st and 2nd difference products according to steps 6. to 8. The PCR according to the MBN reaction (item 7.g) formed an exception - here only 18 cycles in place of 22 ones were carried out.

11. Cloning of the 3rd difference product

The 3rd difference product was initially subjected to a restriction digest with DpnII so as to remove the oligonucleotide adaptors. The reaction product was then applied to a TAE gel and separated electrophoretically. The separated DNA bands were cut out of the gel, the DNA was eluted and cloned into a vector cut with BamHI (pBS Not).

12. Characterization of the difference products

In order to confirm that the cloned DNA fragments were not method artifacts but sequences which were actually included in the investigated DNA representations, Southern analyses were carried out in which the investigated cDNA representations were hybridized with the radioactively labeled cloning products.

Thereafter, those DNA fragments which proved to be "real" difference products in the Southern analysis, were investigated by means of Northern hybridizations: RNAs from the investigated tissues (whn(+/+) skin-cDNA and nu/nu skin-cDNA) were blotted and hybridized with the radioactively labeled cloning products. By this, the differential expression of these sequences was confirmed in the investigated tissues. An analysis of the sequences yielded the cDNA of fig. 1 according to the invention.

Example 2: Preparation and purification of a (PVP) according to the invention

For the preparation of a (PVP) according to the invention, the vector pBSNot-PVP of Example 1 is cleaved by BamHI, the DNA coding for (PVP) is isolated and inserted in the expression vector pQE-8 (Quiagen company) cleaved by BamHI. The expression plasmid pQ/PVP is obtained. Such a plasmid

codes for a fusion protein comprising 6 histidine residues (N terminus partner) and the (PVP) of fig. 1 according to the invention (C terminus partner). pQ/PVP is used for transforming E. coli SG 13009 (cf. Gottesman, S. et al., J. Bacteriol. 148, (1981), 265-273). The bacteria are cultivated in an LB broth with 100 µg/ml ampicillin and 25 µg/ml kanamycin and induced with 60 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) for 4 h. The addition of 6 M guanidine hydrochloride serves for achieving lysis of the bacteria, thereafter a chromatography (Ni-NTA resin) is carried out with the lysate in the presence of 8 M urea corresponding to the instructions of the manufacturer (Quiagen company) of the chromatography material. The bound fusion protein is eluted in a buffer having pH 3.5. After its neutralization, the fusion protein is subjected to an 18 % SDS-polyacrylamide gel electrophoresis and dyed with Coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J. Mol. Biol. 149 (1975), 709-733).

In this way, a (fusion) protein according to the invention can be prepared in highly pure form.

Example 3: Preparation and detection of an antibody according to the invention

A fusion protein of Example 1 according to the invention is subjected to an 18 % SDS-polyacrylamide gel electrophoresis. After dyeing the gel using 4 M sodium acetate, an about 25 kD long band is cut out of the gel and incubated in phosphate-buffered common salt solution. Gel pieces are sedimented before the protein concentration of the supernatant is determined by SDS-polyacrylamide gel electrophoresis, which is followed by Coomassie blue dyeing. Animals are immunized with the gel-purified fusion protein as follows:

Immunization protocol for polyclonal antibodies in rabbits

35 µg gel-purified fusion protein are used per immunization in 0.7 ml PBS and 0.7 ml complete Freund's adjuvant and incomplete Freund's adjuvant, respectively.

day 0: 1st immunization (complete Freund's adjuvant)
day 14: 2nd immunization (incomplete Freund's adjuvant: icFA)
day 28: 3rd immunization (icFA)
day 56: 4th immunization (icFA)
day 80: bleeding to death

The serum of the rabbit is tested in an immunoblot. For this purpose, a fusion protein of Example 1 according to the invention is subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10 (1984), 203-209). The Western blot analysis is carried out as described in Bock, C.-T. et al., Virus Genes 8 (1994), 215-229). For this purpose, the nitrocellulose filter is incubated with a first antibody at 37°C for 1 h. This antibody is the serum of the rabbit (1:10000 in PBS). After several wash steps using PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is a monoclonal goat anti-rabbit-IgG antibody coupled with alkaline phosphatase (Dianova company) (1:5000) in PBS. A 30-minute incubation at 37°C is followed by several wash steps using PBS and then by the alkaline phosphatase detection reaction using developer solution (36 µM 5'-bromo-4-chloro-3-indolylphosphate, 400 µM nitro blue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature, until bands are visible.

In this way, the polyclonal antibodies according to the invention can be prepared.

Immunization protocol for polyclonal antibodies in chickens

40 µg gel-purified fusion protein are used per immunization in 0.8 ml PBS and 0.8 ml complete Freund's adjuvant and incomplete Freund's adjuvant, respectively.

day 0: 1st immunization (complete Freund's adjuvant)
day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
day 50: 3rd immunization (icFA)

Antibodies are extracted from yolk and tested in a Western blot. Polyclonal antibodies according to the invention are detected in this way.

Immunization protocol for monoclonal mouse antibodies

12 µg gel-purified fusion protein are used per immunization in 0.25 ml PBS and 0.25 ml complete Freund's adjuvant and incomplete Freund's adjuvant, respectively. The fusion protein was dissolved in 0.5 ml (without adjuvant) in the 4th immunization.

day 0: 1st immunization (complete Freund's adjuvant)
day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
day 56: 3rd immunization (icFA)
day 84: 4th immunization (PBS)
day 87: fusion

Supernatants of hybridomas are tested in a Western blot. Monoclonal antibodies according to the invention are detected in this way.

Claims

1. A protease-related protein, the protein comprising the amino acid sequence of fig. 1 or an amino acid sequence differing therefrom by one or more amino acids.

2. A DNA coding for a protein according to claim 1, wherein the DNA comprises:
   (a) the DNA of fig. 1 or a DNA differing therefrom by one or more base pairs,
   (b) a DNA hybridizing with the DNA of (a), or
   (c) a DNA related to the DNA of (a) or (b) via the degenerated genetic code.

3. An expression plasmid comprising the DNA according to claim 2.

4. A transformant containing the expression plasmid according to claim 3.

5. A process for the preparation of the protein according to claim 1, comprising the cultivation of the transformant according to claim 4 under suitable conditions.

6. Antibodies directed against the protein according to claim 1.

7. Use of the protein according to claim 1 and the DNA according to claim 2 as well as the antibody according to claim 6 for detecting the keratinization of hair.

8. Use of the protein according to claim 1 for the negative regulation of the keratinization of hair.

9. Use according to claim 8, wherein the protein is present as such or in the form of a nucleic acid expressing it.

10. Use according to claim 8 or 9, wherein substances are also used which inhibit the proteins Ha3 and/or CK15.

11. Use according to claim 10, wherein the substances are antibodies directed against Ha3 and CK15, respectively, and/or anti-sense oligonucleotides, all of which inhibit the expression of the nucleic acids encoding these proteins.

12. Use of the protein according to claim 1 for the positive regulation of the certification of hair.

13. Use according to claim 12, wherein the protein is present in the form of a substance inhibiting it.

14. Use according to claim 13, wherein the substance is an antibody according to claim 6 and/or an anti-sense oligonucleotide which inhibits the expression of the nucleic acid encoding the protein.

15. Use according to any one of claims 12 to 14, wherein the proteins Ha3 and/or CK15 are also present as such or in the form of nucleic acids expressing them.

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5'- TAG GTG GTG TCA TTC CCC TCC AAC CTG AGT GCT GGC AGG TAC  42

                          M   P   M   K   M   L   T   M    8
    ACT GCT GGC CAC CAG CAG ATG CCC ATG AAG ATG CTG ACA ATG   84

    K   M   L   A   L   C   L   V   L   A   K   S   A   W   22
    AAG ATG CTG GCC CTG TGC TTG GTT CTT GCT AAA TCA GCC TGG  126

    S   E   E   Q   E   K   V   V   H   G   G   P   C   L   36
    TCG GAG GAA CAG GAG AAG GTG GTT CAT GGA GGC CCG TGT TTG  168

    K   D   S   H   P   F   Q   A   A   L   Y   T   S   G   50
    AAG GAC TCC CAC CCT TTC CAG GCT GCC CTC TAC ACC TCA GGT  210

    H   L   L   C   G   G   V   L   I   D   P   Q   W   V   64
    CAC TTG CTG TGT GGT GGG GTC CTC ATT GAC CCA CAG TGG GTG  252

    L   T   A   A   H   C   K   K   P   N   L   Q   V   I   78
    CTG ACA GCT GCC CAC TGC AAA AAA CCG AAT CTG CAG GTG ATC  294

    L   G   K   H   N   L   R   Q   T   E   T   F   Q   R   92
    TTG GGG AAA CAC AAC CTA CGG CAA ACA GAG ACT TTC CAA AGG  336

    Q   I   S   V   D   R   T   I   V   H   P   R   Y   N  106
    CAA ATC TCA GTG GAC AGG ACT ATT GTC CAT CCC CGC TAC AAC  378

    P   E   T   H   D   N   D   I   M   M   V   H   L   K  120
    CCT GAA ACC CAC GAC AAT GAC ATC ATG ATG GTG CAT CTG AAA  420

    N   P   V   K   F   S   K   K   I   Q   P   L   P   L  134
    AAT CCA GTC AAA TTC TCT AAA AAG ATC CAG CCT CTG CCC TTG  462

    K   N   D   C   S   E   E   N   P   N   C   Q   I   L  148
    AAG AAT GAC TGC TCT GAG GAG AAT CCC AAC TGC CAG ATC CTG  504

    G   W   G   K   M   E   N   G   D   F   P   D   T   I  162
    GGC TGG GGC AAG ATG GAA AAT GGT GAC TTC CCA< GAT ACC ATT  546

    Q   C   A   D   V   H   L   V   P   R   E   Q   C   E  176
    CAG TGT GCT GAT GTC CAT CTG GTG CCC CGG GAG CAG TGT GAG  588

    R   A   Y   P   G   K   I   T   Q   S   M   V   C   A  190
    CGT GCC TAC CCT GGC AAG ATC ACC CAG AGC ATG GTG TGC GCA  630

    G   D   M   K   E   G   N   D   S   C   Q   G   D   S  204
    GGC GAC ATG AAA GAA GGC AAC GAT TCC TGT CAG GGT GAT TCT  672

    G   G   P   L   V   C   G   G   R   L   R   G   L   V  218
    GGA GGT CCC CTA GTA TGT GGG GGT CGC CTC CGA GGG CTC GTG  714
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Fig. 1

Fig. 1 continued

S	W	G	D	M	P	C	G	S	K	E	K	P	G	232
TCA	TGG	GGT	GAC	ATG	CCC	TGT	GGA	TCA	AAG	GAG	AAG	CCA	GGA	756
V	Y	T	D	V	C	T	H	I	R	W	I	Q	N	246
GTT	TAC	ACC	GAT	GTC	TGC	ACT	CAT	ATC	AGA	TGG	ATC	CAA	AAC	798
I	L	R	N	K	W	L								253
ATC	CTC	AGA	AAC	AAG	TGG	CTG	TGA	-3′						840